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Activation and inhibition of purified skeletal muscle calcium release channel by NO donors in single channel current recordings

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Abstract

The actions of the nitric oxide (NO) donors 1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3 methyl-1-triazine (NOC-7), *S*-nitrosoacetylcysteine (CySNO) and *S*-nitrosoglutathione (GSNO) on the purified calcium release channel (ryanodine receptor) of rabbit skeletal muscle were determined by single channel current recordings. In addition, the activation of the NO donor modulated calcium release channel by the sulfhydryl oxidizing organic mercurial compound 4-(chloromercuri)phenylsulfonic acid (4-CMPS) was investigated. NOC-7 (0.1 and 0.3 mM) and CySNO (0.4 and 0.8 mM) increased the open probability (P_o) of the calcium release channel at activating calcium concentrations (20–100 μM Ca^{2+}) by 60–100%, with no effect on the current amplitude; this activation was abolished by the specific sulfhydryl reducing agent DTT. High concentrations of CySNO (1.6–2 mM) decreased P_o. Activation by GSNO (1 mM) was observed in two thirds of the experiments, but 2 mM and 4 mM GSNO markedly reduced P_o at activating Ca^{2+} (20–100 μM). In contrast to 4-CMPS, NOC-7 or GSNO had no effect at subactivating free Ca^{2+} (0.6 μM). 4-CMPS further increased the open probability of NOC-7- or CySNO-stimulated channels and reversed transiently the reduced open probability of CySNO or GSNO inhibited channels at activating free Ca^{2+}. High concentrations of GSNO did not prevent channel activation of 4-CMPS at subactivating free Ca^{2+}. The NOC-7-, CySNO- or GSNO-modified channels were completely blocked by ruthenium red. It is suggested that nitrosylation/oxidation of sulfhydryls by NO donors and oxidation of sulfhydryls by 4-CMPS affect different cysteine residues essential in the gating of the calcium release channel.

Keywords: Skeletal muscle; Calcium release channel; Ryanodine receptor; Nitric oxide donor; Sulfhydryl nitrosylation; Sulfhydryl oxidation

1. Introduction

Calcium release in skeletal muscle occurs via the calcium release channel (ryanodine receptor) located in the terminal cisternae of the sarcoplasmic reticulum [1–3]. The skeletal muscle calcium release channel (RyR-1) is a homotetramer [4,5] and its activity is regulated by various endogenous effectors (calcium, magnesium, ATP, calmodulin, FK-506-binding protein, sorcin, phosphorylation) and exogenous effectors [6–10]. Nitric oxide (NO) is produced in cardiac

Abbreviations: HSR, heavy sarcoplasmic reticulum; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, 1,4-dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; NOC-7, [1-hydroxy-2-oxo-3-(*N*-methyl-3-aminopropyl)-3 methyl-1-triazine]; GSNO, *S*-nitrosoglutathione; CySNO, *S*-nitroso-*N*-acetylcysteine; 4-CMPS, 4-(chloromercuri)phenylsulfonic acid; pCMB, 4-chloromercuribenzoic acid; SNAP, *S*-nitroso-*N*-acetylpenicillamine

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muscle and skeletal muscle physiologically [11,12]. Since calcium release from the sarcoplasmic reticulum (HSR) initiates muscle contraction in skeletal muscle [13–15] it has been postulated that endogenous NO alters the contractile state possibly by affecting the calcium release channel of sarcoplasmic reticulum [11,12].

Effects of NO donor compounds on the calcium release channel of sarcoplasmic reticulum of skeletal muscle [16–18] and of cardiac muscle [19,20] have been reported. Mezaros et al. [16] found a decrease in the open probability (P_o) with *S*-nitroso-*N*-acetylpenicillamine (SNAP) on the skeletal muscle calcium release channel in HSR, whilst Stoyanovsky et al. [17] reported an increase in P_o with SNAP and *S*-nitrosoacetylcysteine (CySNO) in single channel recordings of skeletal muscle HSR vesicles. Aghadasi et al. [18] postulated that NO donors have no activating effect on the calcium release channel of skeletal muscle HSR at low concentrations, but prevented oxidation of regulatory sulfhydryls, whilst high concentrations of NO donors activated the calcium release channel by producing intersubunit cross-links. Xu et al. [20] demonstrated that *S*-nitrosoacetylcysteine and *S*-nitrosoglutathione (GSNO) activated the purified cardiac calcium release channel, which was associated with both nitrosylation and oxidation of sulfhydryls of calcium release channel subunits. Alkylation or oxidation of sulfhydryls of the calcium release channel had a marked effect on the activity of the calcium release channel [21,22]. Compounds such as 4-(chloromercuri)phenylsulfonic acid (4-CMPS) [23,24], thimerosal [25,26] or the dihydrodipyridines 2,2-DTDP or 4,4-DTDP [26–28] activated the calcium release channel at low concentrations whilst high concentrations inhibited the channel.

The aim of the present investigation was to study firstly the effect of NO donors on the purified skeletal muscle calcium release channel to exclude interference with other proteins present in heavy sarcoplasmic reticulum fractions, and secondly to compare the effect of NO donors causing possibly nitrosylation and oxidation of sulfhydryls of the calcium release channel with those of 4-CMPS which only oxidizes sulfhydryls.

This report demonstrates that NO donors are able to activate and/or inhibit the purified skeletal muscle calcium release channel, similarly as observed by sulfhydryl oxidizing agents. However, activation or inhibition of the calcium release channel by NO donors is modified or reversed by 4-CMPS, indicating that oxidation by 4-CMPS involves different reactive sulfhydryls.

2. Materials and methods

2.1. Materials

GSNO, 4-CMPS, MOPS, HEPES, Tris, histidine, CsCl (ultra pure), ruthenium red, *N*-acetyl-L-cysteine, leupeptin, pepstatin, antipain, phenylmethylsulfonyl fluoride were purchased from Sigma-Aldrich (Vienna, Austria); [^{3}H]ryanodine from Dupont New England Nuclear (Boston, MA, USA); ryanodine from Agrisystems International (Wind Gap); NOC-7 from Alexis (San Diego, CA, USA); phosphatidylserine, phosphatidylethanolamine and phosphatidylcholine from Avanti Polar Lipids (Alabaster, AL, USA); Delrin bilayer chambers (CD22-200; CD13-200) from Warner Instrument (Hamden, NJ, USA). Aprotinin was a generous gift from Bayer Austria (Vienna, Austria). L-*S*-Nitroso-*N*-acetylcysteine (CySNO) was synthesized from L-*N*-acetylcysteine and $NaNO_2$ according to Feelisch and Stamler [29]. Separation of CySNO from *N*-acetylcysteine was carried out by reversed-phase HPLC on a LiChrospher 100 RP-18 column (C-18 column, 0.4×25 cm; Merck, Darmstadt, Germany) with UV detection (L-4200 UV Vis detector; Merck) at 335 nm [30]; the mobile phase consisted of 20% methanol and 30 mM phosphate buffer (pH 3.0). The retention time of CySNO was 13.3 ± 0.06 min (means ± S.E.M; $n = 28$). NOC-7 was dissolved in 0.1 N NaOH. CySNO was kept in 20% methanol, 30 mM phosphate buffer and neutralized with 2 M Tris immediately before use. All other reagents were dissolved in MilliQ deionized water.

2.2. Preparation of sarcoplasmic reticulum

Heavy sarcoplasmic reticulum vesicles (HSR) from rabbit skeletal muscle were prepared as described previously [24]. Briefly, white back muscle (fast twitch muscle) was homogenized in a Waring blender

for 1.5 min in a medium containing 10 mM histidine buffer (pH 7.0) and 100 mM NaCl, and centrifuged for 35 min at 4000×*g*. The supernatant was filtered through cheese cloth and centrifuged for 30 min at 30 000×*g*. The pellet was resuspended in 10 mM histidine buffer (pH 7.0), 0.6 M KCl, 250 mM sucrose and centrifuged for 35 min at 100 000×*g*. The pellet was washed once in a medium containing 10 mM histidine buffer (pH 7.0), 100 mM NaCl, 200 mM sucrose, centrifuged again for 35 min at 100 000×*g* and stored at −80°C or used immediately for the purification of the ryanodine receptor-calcium release channel. All buffers used for the preparation and resuspension of HSR contained 0.5 μg/ml leupeptin, 1 μg/ml antipain, 1.4 μg/ml aprotinin, 1 μM pepstatin, 0.1 mM PMSF, 1 mM benzamidine.

2.3. Preparation of calcium release channel (ryanodine receptor)

The calcium release channel of the terminal cisternae of sarcoplasmic reticulum vesicles was prepared as described previously [24], a slight modification of the preparation used previously [31]. Briefly, heavy sarcoplasmic reticulum vesicles from rabbit skeletal muscle (prepared as above) were solubilized with CHAPS (medium: 40 mM MOPS/Tris (pH 7.0), 1 M NaCl, 2 mM DTT, 1% CHAPS, 0.25% phosphatidylcholine, 0.5 μg/ml leupeptin, 1 μg/ml antipain, 1.4 μg/ml aprotinin, 1 μM pepstatin, 0.1 mM PMSF, 1 mM benzamidine, 15 mg HSR/ml; incubation: 60 min at 3–4°C), followed by centrifugation twice for 35 min at 103 000×*g* (Beckman 65 rotor). The supernatant was centrifuged through a linear 7.5–20% sucrose gradient equilibrated in 40 mM MOPS/Tris (pH 7.0), 300 mM NaCl, 2 mM DTT, 0.5% CHAPS, 0.25% phosphatidylcholine, 0.5 μg/ml leupeptin, 1 μg/ml antipain, 1.4 μg/ml aprotinin, 1 μM pepstatin, 0.1 mM PMSF, 1 mM benzamidine for 14 h at 2°C (Beckman SW28 rotor; 38 ml tubes). Fractions containing the ryanodine receptor (determined by SDS-PAGE) were pooled and dialyzed for 24 h in a medium containing 40 mM MOPS/Tris (pH 7.0), 100 mM NaCl, 2 mM DTT, 0.15 mM $CaCl_2$, 0.1 mM EGTA, 0.5 μg/ml leupeptin, 1 μg/ml antipain, 1.4 μg/ml aprotinin, 1 μM pepstatin, 0.1 mM PMSF, 1 mM benzamidine. Sucrose (200 mM final concentration) was added to the proteoliposomes before storage at −78°C. Preparation and dialysis were carried out at 2–4°C.

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in 5% polyacrylamide gels (0.75 mm thickness) with 3% stacking gels as described previously [31]. The purified ryanodine receptor was incubated with NOC-7 (0.3–0.6 mM), HEPES/Tris (pH 7.4) for 30 min at 25°C followed by addition of 2% SDS, 10% glycerol, 10 mM Tris (pH 6.8). Gels were run without a thiol reducing agent, stained with 0.05% Coomassie blue in 10% acetic acid. Molecular weight standards were run on two separate lanes of the same gel: ferritin (440 000), thyroglobulin (330 000), myosin (212 000).

2.5. Single channel recordings

Single channel recordings were carried out following incorporation of purified calcium release channels (ryanodine receptors) into planar lipid bilayers, essentially according to Coronado et al. [32] as described previously [24,33]. Planar lipid bilayers were formed from phosphatidylserine (10 mg/ml) and phosphatidylethanolamine (10 mg/ml) in decane (Avanti Polar Lipids). The lipid solution was spread over a 200 μm diameter aperture in a delrin cup (Warner Instrument) separating two aqueous compartments. The *cis* bath solution (2.6 ml) and the *trans* bath solution (4 ml) were connected to the head stage input of a model EPC-9 amplifier (Heka Elektronik, Lambrecht, Germany) via Ag/AgCl electrodes and CsCl-agar bridges. The *trans* bath was held at virtual ground. Cs^+ was used as the charge carrier through the calcium release channel to increase the conductance of the channel [32]. The *cis* solution was composed of 10 mM HEPES/Tris (pH 7.4), 480 mM CsCl, and 100 μM $CaCl_2$ or 100 μM $CaCl_2$ plus 80 μM EGTA (free calcium 20 μM). The *trans* solution was composed of 10 mM HEPES/Tris (pH 7.4) and 50 mM CsCl, and in some experiments additionally 100 μM $CaCl_2$ or 100 μM $CaCl_2$ plus 80 μM EGTA (free calcium 20 μM). In experiments with NOC-7 (dissolved in 0.1 N NaOH) the HEPES/Tris buffer was increased to 20 mM or 40 mM to maintain the desired pH of 7.4. Unless stated other-

wise, purified calcium release channels and other reagents were added to the *cis* chamber. Recordings were filtered at 4 kHz with a low-pass Bessel filter, digitized at 40 kHz and stored on the hard disc of a McIntosh/PC. Single channel events were identified using TAC V2.5 software (Skalar Instruments, Seattle, WA, USA). Mean open probability (P_o) of channels were identified by a 50% threshold analysis. The lifetimes of open and closed events were determined by the method of maximum likelihood (TACFit software, Skalar Instruments).

2.6. [^{3}H]Ryanodine binding

Test samples and controls were assayed in triplicate for 90 min at 37°C in 0.2 ml solution containing 20 mM MOPS/Tris (pH 7.0), 0.5 M CsCl, 100 μM $CaCl_2$, 0.1 mg HSR, 0.5 μg/ml leupeptin, 1.4 μg/ml aprotinin, 0.1 mM PMSF and 10 nM [^{3}H]ryanodine [24]. Samples were filtered on glass-fiber filters (presoaked in 1% polyethylene imine) and washed with 10 ml of 20 mM MOPS/Tris (pH 7.0), 1 M NaCl; non-specific binding was measured in the presence of 100 μM unlabeled ryanodine as described previously [24].

2.7. Protein assay

Protein was measured by the Folin method [34] and in the presence of detergents plus phosphatidylcholine, according to Kaplan and Pedersen [35], standardized against bovine serum albumin.

2.8. Calculations

Statistical analysis was carried out by *t*-test using Sigmastat 2 software (Jandel, San Rafael, CA, USA). Averaged results are presented as means ± S.E.M.

3. Results

3.1. Effect of NOC-7 on the purified calcium release channel at activating (20–100 μM) and subactivating (0.6 μM) Ca^{2+} concentrations

An example of the activating effect of NOC-7 on the purified calcium release channel at 20 μM activating Ca^{2+} is illustrated in Fig. 1. 0.1 mM and 0.3 mM NOC-7 added sequentially to the *cis* side (the cytoplasmic side) increased the open probability concentration dependently (Fig. 1A–C, Table 1). Statistical analysis of experiments with NOC-7 showed that the open probability increased significantly from 0.45 ± 0.03 (control, $n = 9$) to 0.62 ± 0.03 (0.1 mM NOC-7, $n = 9$) and to 0.72 ± 0.02 (0.3 mM NOC-7; $n = 6$; means ± S.E.M; Table 1A). The channel activation by 0.3 mM NOC-7 was reversed by addition of 8 mM DTT (Fig. 1D, Table 1B). Subsequent addition of 7 μM ruthenium red completely closed the calcium release channel (Fig. 1E). In four experiments 0.3 mM NOC-7 increased the open probability from 0.40 ± 0.05 (control) to 0.72 ± 0.04 and was reduced to 0.47 ± 0.06 by 8 mM or 16 mM DTT ($n = 4$; means ± S.E.M.; Table 1B), which strongly indicates that the activating effect of the NO donor is due to a modulation of sulfhydryl groups essential for the gating of the calcium release channel. A series of control experiments were carried out in the absence or presence of 2–16 mM DTT. DTT had no significant effect on P_o compared to controls (Table 2A), but 16 mM DTT reduced the open probability about 18%. It is noted, however,

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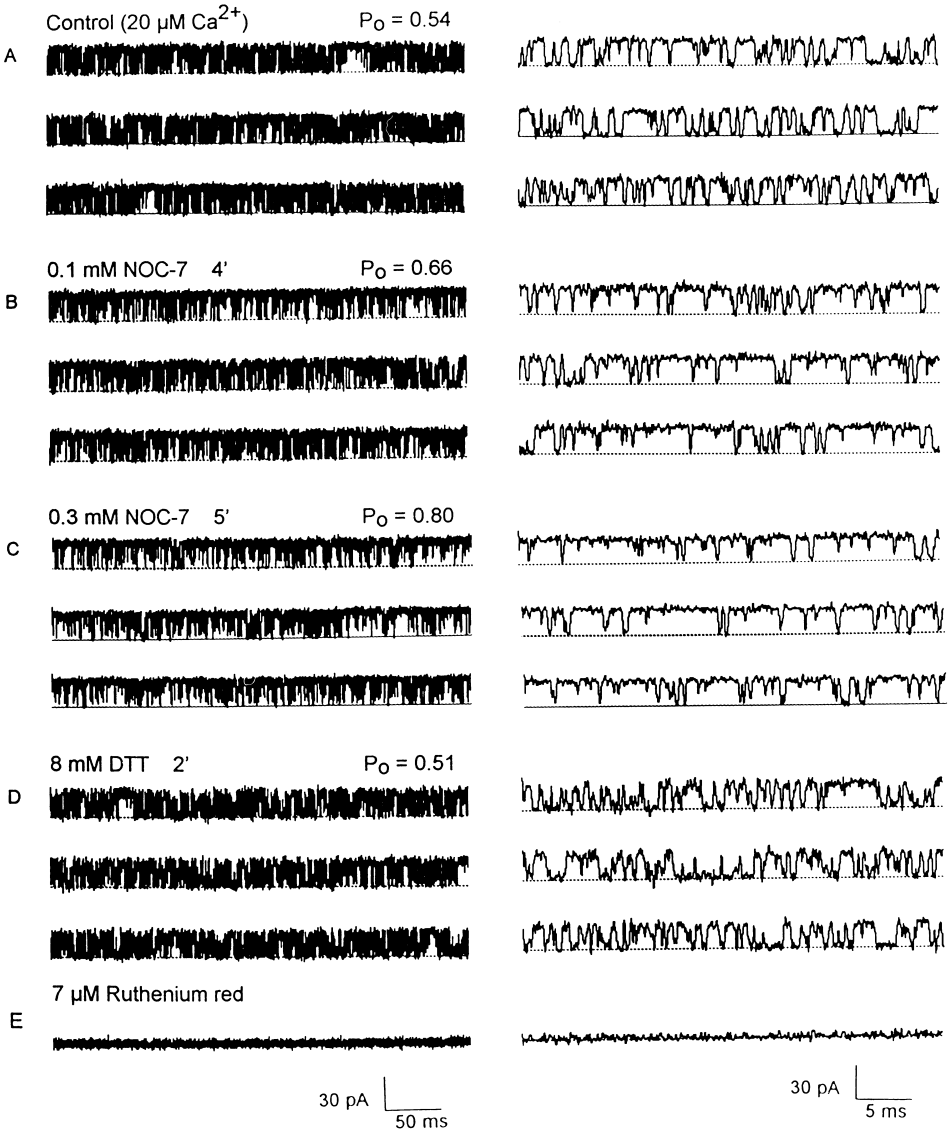
Fig. 1. Activation of single purified skeletal muscle calcium release channel by NOC-7 at 20 μM activating calcium and reversibility by DTT. Single channel currents, shown as upward deflections, were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). The baselines are indicated by the dotted lines. Control and test records (A–E) are from the same channel. Left panel: 400 ms sweeps. Right panel: first 40 ms of each 400 ms sweep. (A) Control: 20 μM Ca^{2+}, $P_o = 0.54$. (B) 0.1 mM NOC-7 added to the *cis* side, $P_o = 0.66$ (4 min). (C) 0.3 mM NOC-7 added to the *cis* side, $P_o = 0.80$ (5 min). (D) 8 mM DTT added to the *cis* side, $P_o = 0.51$. (E) 7 μM ruthenium red. Calibration bars represent 30 pA and 50 ms or 5 ms. Lifetime histograms of open times and channel opening time constants (τ_o) of control and of the same channel modified by 0.3 mM NOC-7 are displayed in F and G. The solid lines represent a fit according to three exponentials. Channel open probabilities (P_o) and τ_o were calculated from 45 600 events (control), 41 600 events (0.1 mM NOC-7), 46 900 events (0.3 mM NOC-7) and 25 600 (8 mM DTT).


Control (20 μM Ca2+)
Po = 0.54
A
0.1 mM NOC-7 4'
Po = 0.66
B
0.3 mM NOC-7 5'
Po = 0.80
C
8 mM DTT 2'
Po = 0.51
D
7 μM Ruthenium red
E
30 pA
50 ms
30 pA
5 ms

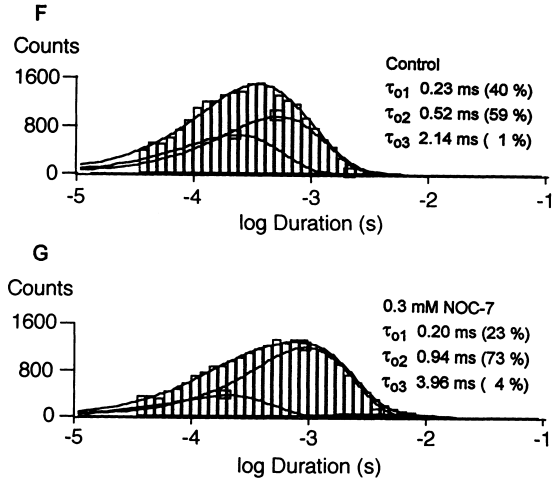

F
Counts
1600
800
0
-5
-4
-3
-2
-1
log Duration (s)
Control
τo1 0.23 ms (40 %)
τo2 0.52 ms (59 %)
τo3 2.14 ms (1 %)
G
Counts
1600
800
0
-5
-4
-3
-2
-1
log Duration (s)
0.3 mM NOC-7
τo1 0.20 ms (23 %)
τo2 0.94 ms (73 %)
τo3 3.96 ms (4 %)

Table 1
Activation of single purified skeletal muscle calcium release channels by NOC-7 at 20–100 μM activating Ca^{2+} and reversibility by DTT

		P_o	n
A	Control	0.45 ± 0.03	9
	NOC-7 0.1 mM	0.62 ± 0.03[a]	9
	NOC-7 0.3 mM	0.72 ± 0.02[a]	6
B	Control	0.40 ± 0.05	4
	NOC-7 0.3 mM	0.72 ± 0.04[a]	4
	NOC-7 0.3 mM/8 or 16 mM DTT	0.47 ± 0.06	4
C	Control (100 μM Ca^{2+})	0.442 ± 0.051	4
	Control (0.6 μM Ca^{2+})	0.017 ± 0.011	4
	NOC-7 0.3 mM	0.003 ± 0.001	4
	NOC-7 0.6 mM	0.007 ± 0.002	4
	40–80 μM 4-CMPS	0.583 ± 0.098	3

Single channel currents were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*) in the presence of 20–100 μM Ca^{2+} (*cis*). Control and test records are from the same channel. (A) Activation of single channel current by 0.1 mM and 0.3 mM NOC-7. (B) Reversibility by 8 mM or 16 mM DTT. (C) Effect of NOC-7 on single purified skeletal muscle calcium release channels at subactivating Ca^{2+} (0.6 μM). Single channel recordings with the purified calcium release channel performed at 0.6 μM Ca^{2+} in the absence or presence of 0.3 mM and 0.6 mM NOC-7 followed by 40–80 μM 4-CMPS. Channel open probabilities (P_o) were calculated from 30 000–50 000 events. Values are means ± S.E.M. for the number of experiments given in parentheses.
[a]Significantly different from controls ($P < 0.001$). The half-life time of NO release from NOC-7 is 10 min.

that 16 mM DTT increased the baseline noise indicating that this high DTT concentration affected the lipid bilayer. For this reason [^{3}H]ryanodine binding to HSR in the absence or presence of the reducing agent DTT was performed under equilibrium conditions. [^{3}H]Ryanodine binding to HSR in the presence of 100 μM Ca^{2+} (carried out with 10 nM [^{3}H]ryanodine and in the presence of 0.5 M CsCl at 37°C for 90 min) was not significantly changed by 2–10 mM DTT (Table 2B). In three binding experiments (37°C, 30 min) 16 mM DTT reduced [^{3}H]ryanodine binding to HSR marginally by 11%.

Means of P_o, current amplitudes and the distribution of the open and closed lifetimes of controls and of the same channels modified by 0.3 mM NOC-7 in six experiments are given in Table 3. The increase in the open probability was associated with an increase of the open lifetimes of the NOC-7-modified channel (control: $\tau_{o1} = 0.27 \pm 0.02$ ms (86%); $\tau_{o2} = 0.67 \pm 0.06$ ms (14%); NOC-7: $\tau_{o1} = 0.17 \pm 0.03$ ms (20%); $\tau_{o2} = 0.71 \pm 0.07$ ms (76%); $\tau_{o3} = 2.50 \pm 0.42$ ms (4%), (Table 3, $n = 6$, means ± S.E.M.; see Fig. 1F,G). The current amplitude was not changed by NOC-7 (Table 3).

Oxidation of sulfhydryls of the calcium release channel by a sulfhydryl reacting organic mercurial (4-CMPS) has two distinct effects on the calcium release channel. Low concentrations of 4-CMPS (20 μM) cause a long lasting activation of the calcium release channel, whilst high concentrations (100 μM) cause a rapid, but short lasting increase in the open probability in single channel recordings with the purified calcium release channel followed by an inhibition of the channel [24]. As shown in Fig. 2

Table 2
Effect of DTT on single channel currents of the purified calcium release channel and [^{3}H]ryanodine binding to HSR in the presence of 100 μM activating Ca^{2+} and 0.5 M CsCl

	Open probability (n)
A	
Control	0.55 ± 0.05 (7)
DTT 2 mM	0.57 ± 0.05 (7)[ns]
Control	0.54 ± 0.04 (7)
DTT 4 mM	0.49 ± 0.05 (7)[ns]
Control	0.47 ± 0.04 (7)
DTT 8 mM	0.42 ± 0.04 (7)[ns]
Control	0.44 ± 0.09 (4)
DTT 16 mM	0.36 ± 0.06 (4)[ns]

	[^{3}H]Ryanodine bound (% of control) (n)
B	
DTT 2 mM	94.3 ± 3.1 (6)
DTT 4 mM	98.8 ± 2.3 (6)
DTT 8 mM	102.9 ± 3.0 (6)
DTT 10 mM	100.2 ± 2.5 (5)

(A) Single channel recordings with the purified calcium release channel were performed in the absence or presence of 2–16 mM DTT. Single channel currents were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). 100 μM Ca^{2+} *cis* was the sole activator. Control and test recordings are from the same channel. [ns]Not significantly different from controls. (B) [^{3}H]Ryanodine binding to HSR in the absence or presence of 2–10 mM DTT was carried out at 100 μM activating Ca^{2+}, 20 mM MOPS/Tris (pH 7.0), 0.5 M CsCl, 10 nM [^{3}H]ryanodine and 100 μg/HSR in a volume of 0.2 ml (37°C, 90 min). Values are given as % of controls (± S.E.M.) for the number of experiments given in parentheses. [^{3}H]Ryanodine binding of controls was 4.33 ± 0.62 pmol/mg HSR ($n = 6$).

Table 3
Mean open probability, mean current amplitude and mean open and closed lifetimes of controls and 0.3 mM NOC-7

	Control ($n=6$)		0.3 mM NOC-7 ($n=6$)	
	Means ± S.E.M.	Area (%)	Means ± S.E.M.	Area (%)
P_o	0.43 ± 0.04		0.72 ± 0.02	
Amplitude (pA)	22.4 ± 0.3		21.9 ± 0.4	
Mean T_o (ms)	0.37 ± 0.04		0.73 ± 0.07	
Mean T_c (ms)	0.47 ± 0.05		0.28 ± 0.02	
τ_{o1} (ms)	0.27 ± 0.02	(86)	0.17 ± 0.03	(20)
τ_{o2} (ms)	0.67 ± 0.06	(14)	0.71 ± 0.07	(76)
τ_{o3} (ms)			2.50 ± 0.42	(4)
τ_{c1} (ms)	0.39 ± 0.05	(88)	0.18 ± 0.01	(96)
τ_{c2} (ms)	1.05 ± 0.22	(12)	1.04 ± 0.29	(4)

Channel open probabilities (P_o), mean current amplitude (pA), mean channel open (T_o) and closed (T_c) duration (ms), cumulative mean open and closed channel time constants (τ_o, τ_c) and values of the percent of the channel represented by a time constant for purified calcium release channel activated by 20–100 μM *cis* Ca^{2+} in the absence and presence of 0.3 mM NOC-7. Calcium release channels were recorded at 0 mV voltage holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). Values are means ± S.E.M. from six channels included in the analysis.

the NOC-7-modified calcium release channel ($P_o = 0.67$; Fig. 2B) was markedly activated by 100 μM 4-CMPS ($P_o = 0.95$; Fig. 2C) and then completely blocked.

To test for a possible activating effect of NOC-7 at low free calcium experiments with the purified calcium release channel were carried out at a free calcium concentration of 0.6 μM as illustrated in Fig. 3. Channel open probability (P_o) of the control at 20 μM Ca^{2+} was 0.57 (Fig. 3A). When the free calcium concentration on the *cis* side of the bilayer was reduced to 0.6 μM by addition of EGTA the calcium release channel was predominantly closed ($P_o = 0.002$; Fig. 3B). Sequential addition of 0.3 mM and 0.6 mM NOC-7 to the solution on the *cis* side had no effect on P_o ($P_o = 0.001$, Fig. 3C). However, addition of 80 μM 4-CMPS caused a transient activation the calcium release channel in the presence of 0.6 mM NOC-7 ($P_o = 0.71$, Fig. 3D). Statistical analysis of four experiments, carried out at low free calcium of 0.6 μM, did not reveal any activating effect of NOC-7 at concentrations of 0.3 mM or 0.6 mM (Table 1C). The open probability in the presence of 0.6 μM free calcium and 0.6 mM NOC-7 was 0.007 ± 0.002 ($n=4$; means ± S.E.M.) and increased to 0.58 ± 0.10 following addition of 40–80 μM 4-CMPS ($n=3$; means ± S.E.M.; Table 1C). The effect of 4-CMPS on the NOC-7-modified calcium release channel was very similar to the data on activation and inhibition of the purified calcium release channel by 4-CMPS or pCMB reported previously [24]. However, activation of the NOC-7 modified calcium release channels required usually higher concentrations of 4-CMPS and was followed by a complete closure of the channel, whilst in controls high concentrations of 4-CMPS caused a long lasting low activity state following activation of the calcium release channel [24].

3.2. *Effect of CySNO on the purified calcium release channel at activating (20–100 μM) Ca^{2+} concentrations*

An example of the activating effect of CySNO on the purified calcium release channel at an activating calcium concentration of 60 μM is illustrated in Fig. 4. 0.4 mM and 0.8 mM CySNO added sequentially to the *cis* side increased the open probability concentration dependently (Fig. 4A–C). Statistical analysis of experiments with CySNO showed that the open probability increased significantly from 0.44 ± 0.03 (control, $n=11$) to 0.61 ± 0.04 (0.4 mM CySNO, $n=11$) and to 0.74 ± 0.03 (0.8 mM CysNO; $n=6$; means ± S.E.M; Table 4A). The channel activation by 0.8 mM CySNO was reversed by addition of 16 mM DTT (Fig. 4D), which strongly indicates that the activating effect of CySNO is due to a modulation of sulfhydryl groups essential for the gating of the calcium release channel. In three experiments 0.8 mM CySNO increased the open probability from

0.48 ± 0.05 (control) to 0.75 ± 0.02 and was reduced to 0.42 ± 0.08 by 16 mM DTT ($n = 3$; means ± S.E.M.; Table 4B). Addition of 8 mM DTT to calcium release channels activated by 0.8 mM CySNO did not reduce the increased open probability in two of the three experiments. This finding is similar to the effect of CySNO and DTT on the purified cardiac calcium release channel; the activating effect of CySNO could not be reduced by 10 mM DTT [20].

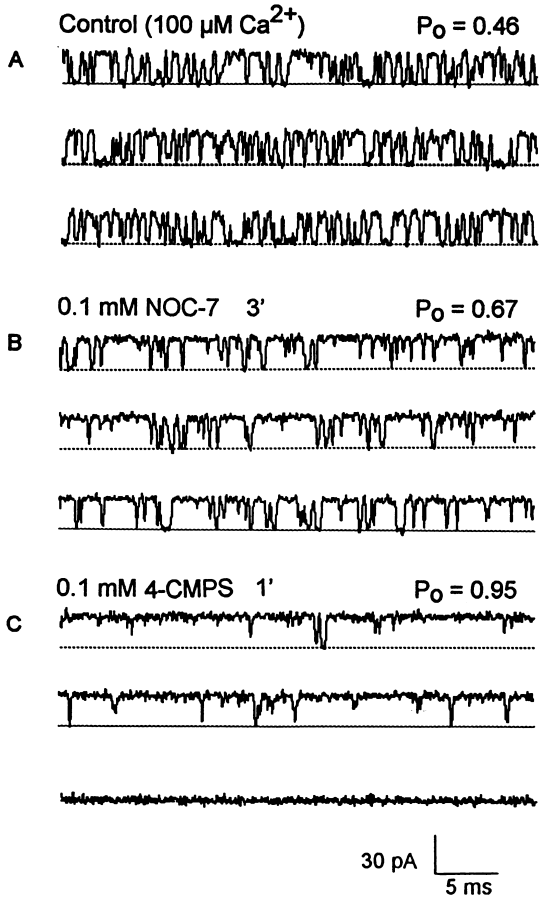


Fig. 2. Activation of single purified skeletal muscle calcium release channel by NOC-7 and 4-CMPS at 100 μM activating Ca^{2+}. Single channel currents, shown as upward deflections, were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). The baselines are indicated by the dotted lines. Control and test records (A–C) are from the same channel. (A) Control: 100 μM Ca^{2+}, $P_o = 0.46$. (B) Activation by 0.1 mM NOC-7 added to the *cis* side, $P_o = 0.67$. (C) 100 μM 4-CMPS added to the *cis* side induced a transient, maximum channel activation followed by closure of the channel within 20 s. Calibration bars represent 30 pA and 5 ms.

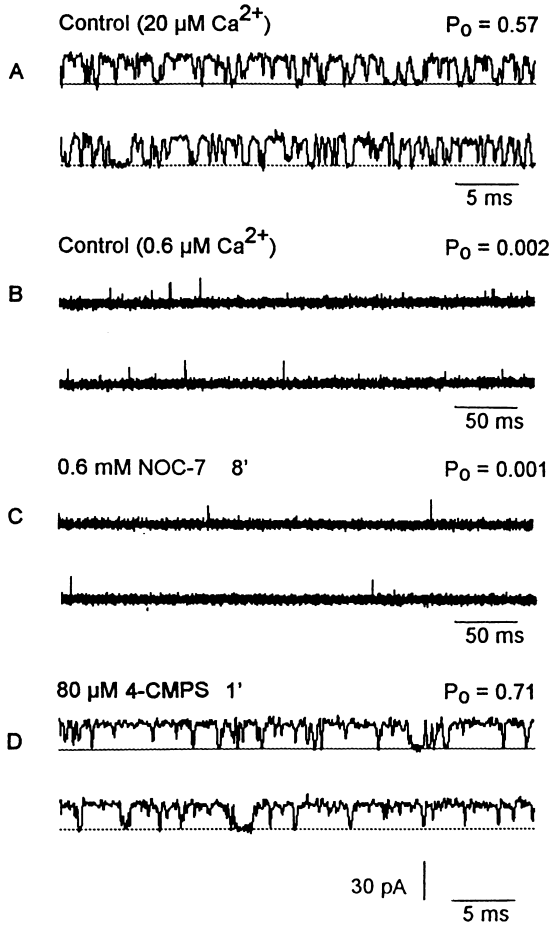


Fig. 3. Effect of NOC-7 and 4-CMPS on single purified skeletal muscle calcium release channels at low free calcium (0.6 μM). Single channel currents, shown as upward deflections, were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). The baselines are indicated by the dotted lines. Control and test records (A–D) are from the same channel. (A) Control: 20 μM Ca^{2+}, $P_o = 0.57$. (B) Control: 0.6 μM Ca^{2+}, $P_o = 0.002$. (C) 0.6 mM NOC-7, $P_o = 0.001$. (D) Activation of the channel by 40 μM 4-CMPS added to the *cis* side, $P_o = 0.71$. Calibration bars represent 30 pA and 5 ms.

Means of P_o, current amplitudes and the distribution of the open and closed lifetimes of controls and of the same channels modified by 0.8 mM CySNO in six experiments are given in Table 5. The increase in the open probability was associated with an increase of the open lifetimes of the CySNO-modified channel (control: $\tau_{o1} = 0.28 \pm 0.02$ ms (81%); $\tau_{o2} = 0.64 \pm 0.07$ ms (19%); CySNO: $\tau_{o1} = 0.36 \pm 0.05$ ms (44%); $\tau_{o2} = 1.05 \pm 0.20$ ms (55%); $\tau_{o3} = 4.55 \pm 1.03$ ms (1%)) (Table 5, $n = 6$, means ± S.E.M.; see Fig.

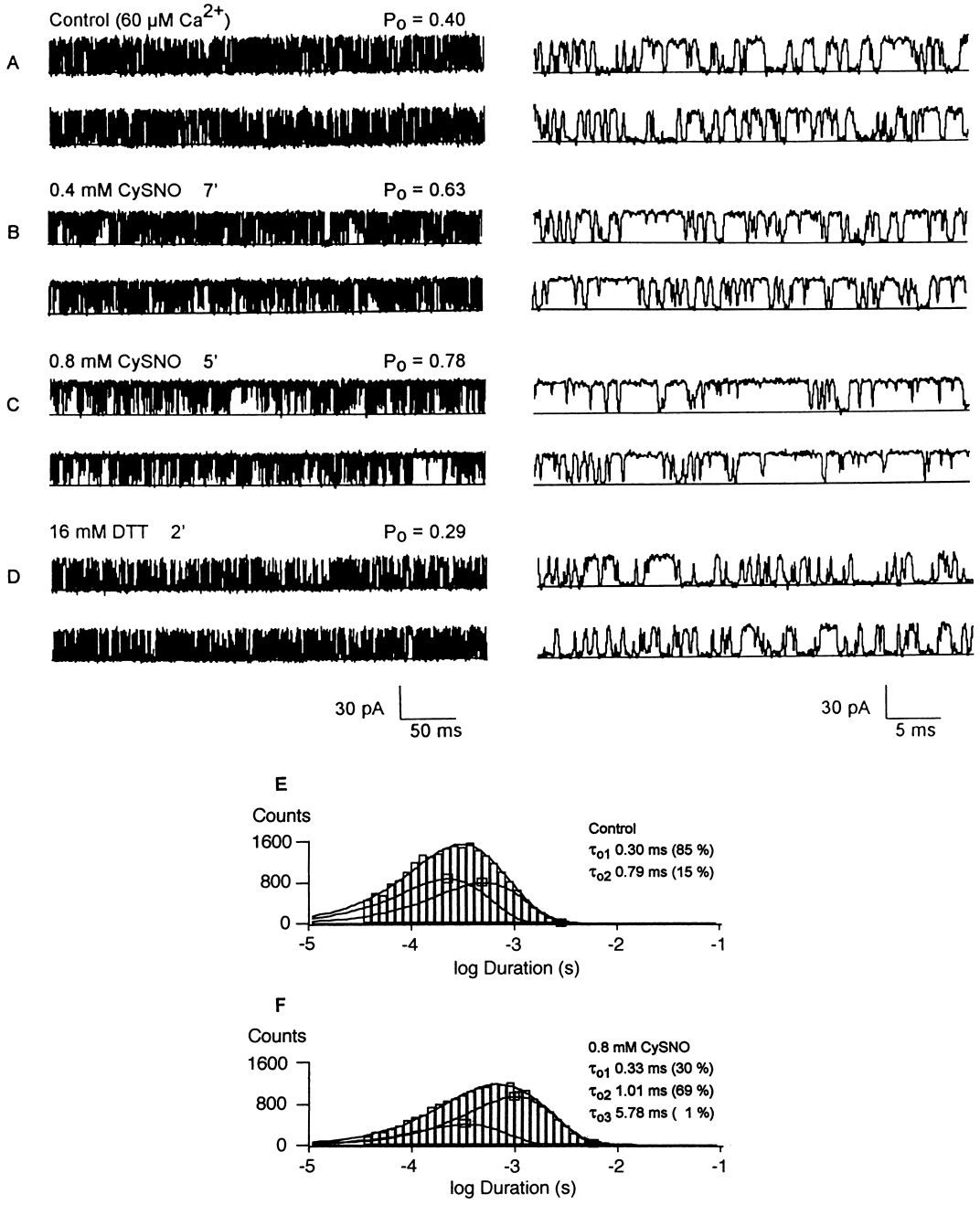


Fig. 4. Activation of single purified skeletal muscle calcium release channel by CySNO at 60 μM activating Ca^{2+} and reversibility by DTT. Single channel currents, shown as upward deflections, were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). The baselines are indicated by the solid lines. Control and test records (A–D) are from the same channel. Left panel: 400 ms sweeps. Right panel: first 40 ms of each 400 ms sweep. (A) Control: 60 μM Ca^{2+}, $P_o = 0.40$. (B) 0.4 mM CySNO added to the *cis* side, $P_o = 0.63$ (7 min). (C) 0.8 mM CySNO added to the *cis* side, $P_o = 0.78$ (5 min). (D) 16 mM DTT added to the *cis* side, $P_o = 0.29$ (2 min). Calibration bars represent 30 pA and 50 ms or 5 ms. Lifetime histograms of open times and channel opening time constants (τ_o) of control and of the same channel modified by 0.8 mM CySNO are displayed in E and F. The solid lines represent a fit according to three exponentials. Channel open probabilities (P_o) and τ_o were calculated from 45 900 events (control), 41 800 events (0.4 mM CySNO), 39 600 events (0.8 mM CySNO) and 11 000 events (16 mM DTT).

Table 4
Effect of CySNO on single purified skeletal muscle calcium release channels at 20–100 μM activating Ca^{2+} and reversibility by DTT

		P_o	n
A	Control	0.44 ± 0.03	11
	CySNO 0.4 mM	0.61 ± 0.04[a]	11
	CySNO 0.8 mM	0.74 ± 0.03[a]	6
B	Control	0.48 ± 0.05	3
	CySNO 0.8 mM	0.75 ± 0.02[a]	3
	CySNO 0.8 mM/16 mM DTT	0.42 ± 0.08	3

Single channel currents were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*) in the presence of 20–100 μM Ca^{2+} (*cis*). Control and test records are from the same channel. (A) Activation of single channel current by 0.4 mM and 0.8 mM CySNO. (B) Reversibility by 16 mM DTT. Channel open probabilities (P_o) were calculated from 30 000–50 000 events. Values are means ± S.E.M. for the number of experiments given in parentheses. [a]Significantly different from controls ($P < 0.001$).

4E,F). The current amplitude was not changed by CySNO (Table 5).

At high concentrations of CySNO (1.6–2.0 mM) the activation by CySNO declined below control levels, i.e. high concentrations of CySNO caused an inhibition of the calcium release channel (Fig. 5). This inhibition could not be reversed by 8 mM or 16 mM DTT (data not shown). However, when 80 μM 4-CMPS were added to the *cis* side solution the CySNO-induced inhibition of the calcium release channel was reversed and the channel was fully activated with an increase in the open probability from 0.28 to 0.94 (Fig. 5C,D). In three experiments with a mean open probability of 0.38 ± 0.02 (control) and activation of the calcium release channel to 0.60 ± 0.03 by 0.8 mM CySNO, a further addition of 1.6–2.0 mM CySNO reduced P_o to 0.24 ± 0.04; addition of 20–80 μM 4-CMPS to the same channels increased the open probability transiently to 0.86 ± 0.06 (means ± S.E.M., $n = 3$).

3.3. Effect of GSNO on the calcium release channel at activating (20–100 μM) and subactivating (0.6 μM) Ca^{2+} concentrations

An example of the effects of 1 and 4 mM GSNO on the purified calcium release channel at 20 μM activating calcium is shown in Fig. 6. 1 mM GSNO gave no straightforward effect on the open probability of the calcium release channel: activation (Fig. 6), no effect or inhibition of the calcium release channel was observed. Activation of the calcium release channel was observed in ten of 17 experiments (control: $P_o = 0.45 \pm 0.04$; 1 mM GSNO: $P_o = 0.63 \pm 0.06$; $n = 10$, means ± S.E.M., Table 6). In seven of 17 experiments 1 mM GSNO had either no effect or caused inhibition of the calcium release channel. The mean values (± S.E.M.) for the open probability in all experiments obtained in the absence of GSNO (controls: $P_o = 0.48 \pm 0.03$; $n = 17$) and in the presence of 1 mM GSNO from the same channels

Table 5
Mean open probability, mean current amplitude and mean open and closed lifetimes of controls and 0.8 mM CySNO

	Control ($n = 6$)		0.8 mM CySNO ($n = 6$)	
	Means ± S.E.M.	Area (%)	Means ± S.E.M.	Area (%)
P_o	0.46 ± 0.04		0.74 ± 0.03	
Amplitude (pA)	22.4 ± 1.2		21.8 ± 1.1	
Mean T_o (ms)	0.41 ± 0.03		0.81 ± 0.11	
Mean T_c (ms)	0.47 ± 0.05		0.27 ± 0.03	
τ_{o1} (ms)	0.28 ± 0.02	(81)	0.36 ± 0.05	(44)
τ_{o2} (ms)	0.64 ± 0.07	(19)	1.05 ± 0.20	(55)
τ_{o3} (ms)			4.55 ± 1.03	(1)
τ_{c1} (ms)	0.36 ± 0.04	(94)	0.18 ± 0.05	(96)
τ_{c2} (ms)	1.47 ± 0.20	(6)	1.92 ± 0.93	(4)

Channel open probabilities (P_o), mean current amplitude (pA), mean channel open (T_o) and closed (T_c) duration, cumulative mean open and closed channel time constants (τ_o, τ_c) and values of the percent of the channel represented by a time constant for purified calcium release channel activated by 0.8 mM CySNO. Calcium release channels were recorded at 0 mV voltage holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). Values are means ± S.E.M. from six channels included in the analysis.

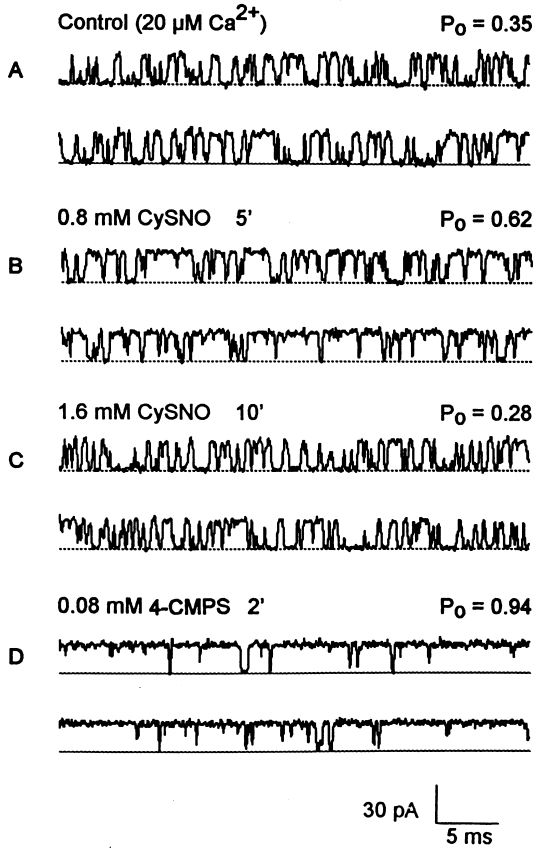


Fig. 5. Activation and inhibition of single purified skeletal muscle calcium release channel by CySNO at 20 μM activating Ca^{2+} and reactivation by 4-CMPS. Single channel currents, shown as upward deflections, were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). The baselines are indicated by the dotted lines. Control and test records (A–D) are from the same channel. (A) Control: 20 μM Ca^{2+}, $P_o = 0.35$. (B) 0.8 mM CySNO added to the *cis* side, $P_o = 0.62$ (5 min). (C) 1.6 mM CySNO added to the *cis* side reduced P_o to 0.28 (10 min). (D) 80 μM 4-CMPS added to the *cis* side, $P_o = 0.94$ (2 min). Channel open probabilities (P_o) were calculated from 30 000 events (control), 30 000 events (0.8 mM CySNO), 38 000 events (1.6 mM CySNO) and 16 000 events (80 μM 4-CMPS). Calibration bars represent 30 pA and 5 ms.

($P_o = 0.53 \pm 0.05$; $n = 17$) were not significantly different (Table 6). However, GSNO concentrations of 2 mM and 4 mM (Fig. 6C) reduced the open probability. GSNO concentrations of 2 mM and 4 mM caused a concentration dependent, significant reduction of P_o. 2 mM GSNO reduced P_o from 0.45 ± 0.03 (control; $n = 11$) to 0.18 ± 0.03 ($n = 11$) and 4 mM GSNO reduced P_o from 0.49 ± 0.03 (control; $n = 10$) to 0.07 ± 0.02 (means ± S.E.M, $n = 10$) (Table 6). The distribution of the open and closed lifetimes of the calcium release channel in the presence of 1 mM and 4 mM GSNO versus control are shown in Fig. 6E–G.

Means of P_o, current amplitudes and the distribution of the open and closed lifetimes of controls and of the same channels modified by 4 mM GSNO in eight experiments are given in Table 7. The decrease in the open probability (control: 0.45 ± 0.04; 4 mM GSNO: 0.051 ± 0.007) was associated with a decrease in the frequency of channel fluctuations and a marked increase in the closed lifetimes of the GSNO modified channel and the best fit of the closed lifetimes was obtained by three exponentials (control: $\tau_{c1} = 0.36 \pm 0.04$ ms (93%); $\tau_{c2} = 1.47 \pm 0.50$ ms (7%); GSNO: $\tau_{c1} = 0.61 \pm 0.10$ (20%), $\tau_{c2} = 2.71 \pm 0.12$ ms (71%) and $\tau_{c3} = 8.04 \pm 1.02$ ms (9%)) (Table 7, $n = 8$, means ± S.E.M.). The open lifetimes (control: $\tau_{o1} = 0.25 \pm 0.17$ ms (71%); $\tau_{o2} = 0.51 \pm 0.06$ ms (29%); GSNO: $\tau_{o1} = 0.11 \pm 0.01$ ms (95%); $\tau_{o2} = 0.68 \pm 0.11$ ms (5%)) were reduced by 4 mM GSNO (Table 7). The current amplitude was not affected by inhibitory concentrations of GSNO (Table 7).

The GSNO-induced inhibition was not changed by addition of 8 mM or 16 mM DTT. However, when 20 μM 4-CMPS were added to the *cis* side of the bathing solution the GSNO-induced inhibition of the calcium release channel was reversed as shown in Fig. 6: P_o decreased from 0.48 (control) to 0.08 in the presence of 4 mM GSNO and increased to 0.90 within 1.5 min after addition of 20 μM 4-CMPS to the *cis* side followed by a complete inactivation of the calcium release channel within 3 min. In experiments carried out under conditions described in Fig. 6, the increase in the open probability by 20–40 μM 4-CMPS was associated with a marked increase in the open lifetimes and the best fit was obtained by three exponentials ($\tau_{o1} = 0.21 \pm 0.05$ ms (29%); $\tau_{o2} = 1.41 \pm 0.52$ ms (51%); $\tau_{o3} = 7.08 \pm 2.75$ ms (20%), Table 7, $n = 4$, means ± S.E.M.). The modulation of the GSNO-inhibited channels by the organic mercurial compound was similar to the data on activation of the purified calcium release channel by 4-CMPS or pCMB reported previously [24]. However, a higher concentration of 4-CMPS was required for the activation of the GSNO-inhibited calcium release channel (40 μM in three of four experiments) as usually necessary in controls (absence of GSNO).

To test for a possible activating effect of GSNO at

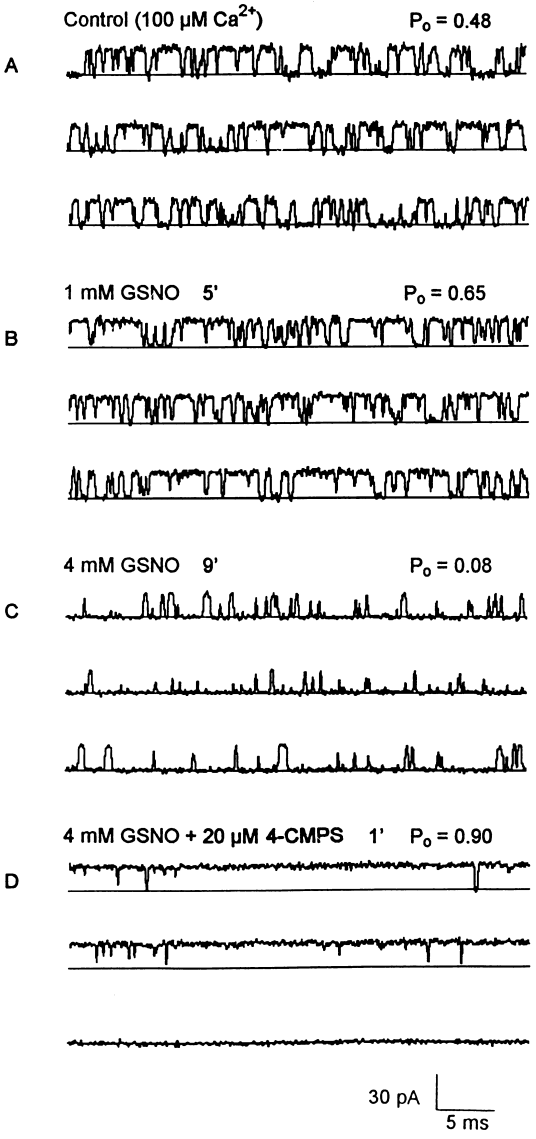


Fig. 6. Activation and inhibition of single purified skeletal muscle calcium release channels at 100 μM activating Ca^{2+} by GSNO and reactivation of the GSNO-inhibited channel by 4-CMPS. Single channel currents, shown as upward deflections, were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). The baselines are indicated by the solid lines. Control and test records (A–D) are from the same channel. (A) Control: 100 μM Ca^{2+}, $P_o = 0.48$. (B) Activation by 1 mM GSNO added to the *cis* side, $P_o = 0.65$ (5 min). (C) Inhibition by 4 mM GSNO added to the *cis* side, $P_o = 0.08$ (9 min). (C) 20 μM 4-CMPS added to the *cis* side induced a transient reactivation of the channel, $P_o = 0.90$ (1 min) which was followed by closure of the channel. Calibration bars represent 30 pA and 5 ms. Lifetime histograms of open and closed times and channel opening and closed time constants (τ_o, τ_c) of control and of the same channel modified by 0.1 mM and 4 mM GSNO are displayed in E–G. Channel open probabilities (P_o) and τ were calculated from 22 700 events (control), 25 600 events (1 mM GSNO), 22 200 events (4 mM GSNO) and 10 400 (4 mM GSNO/20 μM 4-CMPS).

low free calcium similar experiments as described above were carried with the purified calcium release channel at a free calcium concentration of 0.6 μM as illustrated in Fig. 7. Channel open probability (P_o) of the control at 20 μM Ca^{2+} was 0.54 (Fig. 7A). When the free calcium concentration on the *cis* side of the bilayer was reduced to 0.6 μM by addition of EGTA the calcium release channel was predominantly closed ($P_o = 0.02$; Fig. 7B). The addition of 1–4 mM GSNO to the solution on the *cis* side had

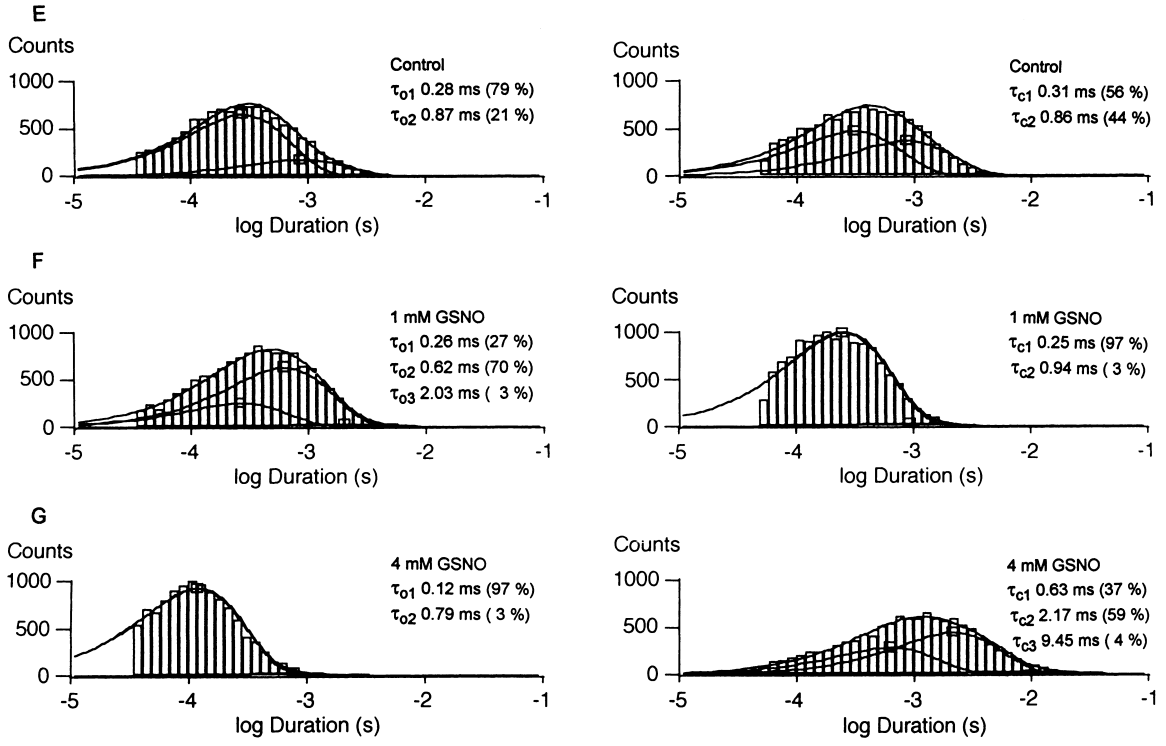


Fig. 6 (*continued*).

little effect on P_o (Fig. 7C and 7D), whilst 20 μM 4-CMPS increased the open probability ($P_o = 0.38$, Fig. 7E). Statistical analysis of experiments carried out at low free calcium of 0.6 μM did not reveal any activating effect of GSNO at concentrations of 1 mM to 4 mM (Table 8). However, 20–40 μM 4-CMPS activated the calcium release channel in the presence of 4 mM GSNO. The open probability in the presence of a low free calcium concentration (0.6 μM) and 4 mM GSNO was 0.044 ± 0.017 ($n = 6$; means ± S.E.M.) and increased to 0.62 ± 0.12 following addition of 20–40 μM 4-CMPS ($n = 3$; means ± S.E.M.; Table 8), i.e. even high concentrations of GSNO did not prevent the activation of the calcium release channel by 4-CMPS.

4. Discussion

The present study demonstrates a concentration-dependent activation of the purified calcium release channel of rabbit skeletal muscle by NOC-7 (Fig. 1, Table 1) and CySNO (Fig. 4, Table 4) in the presence of calcium as sole activator of the calcium release channel. Both NO donors increased the open probability of single channel current fluctuations at 20–100 μM activating Ca^{2+} by 60–100%, without affecting the current amplitude. With GSNO an activating effect on the calcium release channel was seen only in about two thirds of the experiments (ten from 17) at a concentration of 1 mM (Fig. 6) whilst in seven of 17 experiments 1 mM GSNO had either no effect or a slight inhibitory effect (Table 6). The reason for these observations is not clear, but channels may vary in their susceptibility to inhibitory concentrations of GSNO. 2 mM GSNO decreased the open probability of the calcium release channels by about 60% and 4 mM GSNO reduced the open probability about one order of magnitude (Table 6). At high concentrations of CySNO the activating effect declined and the open probability was reduced below control values. No inhibitory effect was observed with NOC-7 at the tested concentrations up to 0.6 mM. Our findings with the purified calcium release channel are in good agreement with the reported activation of the calcium release channel in skeletal muscle HSR vesicles by CySNO [17] and NOC-9 or NOC-15 [18].

Table 6
Effect of GSNO on single purified skeletal muscle calcium release channels at 20–100 μM activating calcium

	P_o	n
Control	0.45 ± 0.04	10
GSNO 1 mM	0.63 ± 0.06[a]	10
Control	0.48 ± 0.03	17
GSNO 1 mM	0.53 ± 0.05	17
Control	0.45 ± 0.03	11
GSNO 2 mM	0.18 ± 0.03[b]	11
Control	0.49 ± 0.03	10
GSNO 4 mM	0.07 ± 0.02[b]	10

Single channel recordings with the purified calcium release channel were performed in the absence or presence of 1, 2 or 4 mM GSNO. Single channel currents were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*) in the presence of 20–100 μM Ca^{2+} (*cis*). Control and test records are from the same channel. Channel open probabilities (P_o) were calculated from 15 000–30 000 events. Values are means ± S.E.M. for the number of experiments given in parentheses. Significantly different from controls: [a] $P = 0.02$, [b] $P < 0.001$.

The increase in the open probability by NOC-7 or CySNO at maximally activating calcium concentrations (20–100 μM) was mainly due to an increase in the mean open channel duration (Figs. 1 and 4). This increase in the open channel duration was due to a shift from short openings to longer openings of the calcium release channel. The best fit of the open lifetimes was obtained by three exponentials (Tables 3 and 5). The decrease in the open probability by CySNO or GSNO at activating calcium concentrations was associated with a decrease in the frequency of the channel openings, a decrease in the mean open channel duration and a marked increase in the mean closed channel duration (Table 7). The best fit of the closed lifetimes in the presence of a high inhibitory GSNO concentration was obtained by three exponentials (Table 7). The tested NO donors had no effect on the current amplitude.

The mercaptide complex formed by binding of 4-CMPS to activating sulfhydryls of the calcium release channel was reversible in the presence of low concentrations of the specific thiol reducing agent DTT [24], but high concentrations of DTT were required to reduce the NOC-7- or CySNO-induced increase in the open probability close to control levels. These findings suggest that the effects of the tested NO donors occurred, at least predominantly, through modification of sulfhydryls of the calcium release channel protein.

The mechanism responsible for the activation of the purified skeletal muscle calcium release channel by the tested NO donors NOC-7, CySNO and GSNO may be due to nitrosylation/oxidation of sulf-

Table 7
Mean open probability, mean current amplitude and mean open and closed lifetimes of controls, 4 mM GSNO, 4 mM GSNO and 20–40 μM 4-CMPS

	Control ($n = 8$)		4 mM GSNO ($n = 8$)		4 mM GSNO+20–40 μM 4-CMPS ($n = 4$)	
	Means ± S.E.M.	Area (%)	Means ± S.E.M.	Area (%)	Means ± S.E.M.	Area (%)
P_o	0.45 ± 0.04		0.051 ± 0.007		0.79 ± 0.06	
Amplitude (pA)	20.8 ± 0.4		19.4 ± 0.6		20.1 ± 0.4	
Mean T_o (ms)	0.42 ± 0.04		0.15 ± 0.01		2.61 ± 1.38	
Mean T_c (ms)	0.45 ± 0.05		3.56 ± 1.03		0.50 ± 0.14	
τ_{o1} (ms)	0.25 ± 0.17	(71)	0.11 ± 0.01	(95)	0.21 ± 0.05	(29)
τ_{o2} (ms)	0.51 ± 0.06	(29)	0.68 ± 0.11	(5)	1.41 ± 0.52	(51)
τ_{o3} (ms)					7.08 ± 2.75	(20)
τ_{c1} (ms)	0.36 ± 0.04	(93)	0.61 ± 0.10	(20)	0.12 ± 0.04	(95)
τ_{c2} (ms)	1.47 ± 0.50	(7)	2.71 ± 0.12	(71)	3.72 ± 1.35	(5)
τ_{c3} (ms)			8.04 ± 1.02	(9)		

Channel open probabilities (P_o), mean channel open (T_o) and closed (T_c) duration, cumulative mean open and closed channel time constants (τ_o, τ_c) and values of the percent of the channel represented by a time constant for purified calcium release channel activated by 20–100 μM *cis* Ca^{2+} ($n = 8$), inhibited by 4 mM GSNO ($n = 8$) and reactivated by 20–40 μM 4-CMPS ($n = 4$). Calcium release channels were recorded at 0 mV voltage holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). Values are means ± S.E.M. for the number of channels included in the analysis.

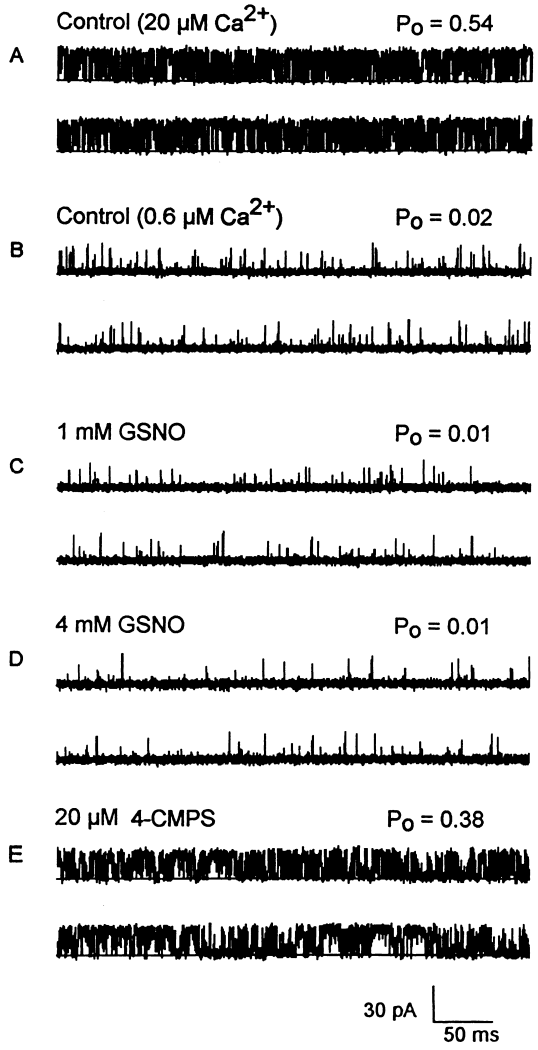


Fig. 7. Effect of GSNO and 4-CMPS on single purified skeletal muscle calcium release channel at low free calcium (0.6 μM). Single channel currents, shown as upward deflections, were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). The baselines are indicated by the solid lines. Control and test records (A–E) are from the same channel. (A) Control: 20 μM Ca^{2+}, $P_o = 0.54$. (B) Control 0.6 μM Ca^{2+}, $P_o = 0.02$. (C) 1 mM GSNO added to the *cis* side (0.6 μM Ca^{2+}), $P_o = 0.01$. (D) 4 mM GSNO (0.6 μM Ca^{2+}), $P_o = 0.01$. (E) Activation of the channel by 20 μM 4-CMPS added to the *cis* side, $P_o = 0.38$. Calibration bars represent 30 pA and 50 ms.

hydryls of cysteine residues essential for the gating of the calcium release channel.

S-Nitrosylation or poly-*S*-nitrosylation of proteins by NO donors has been shown [20,36]. For the purified cardiac calcium release channel it has been elegantly demonstrated that the activation of the channel by CySNO and GSNO was associated with nitrosylation and oxidation of sulfhydryls of the calcium release channel protein [20]. Nitrosylation and oxidation of the calcium release channel were concentration-dependent and occurred simultaneously [20].

The sulfhydryl oxidizing organic mercurial compound 4-CMPS, which was used for comparison, showed different effects on the calcium release channel as observed in the presence of NO donors and, in addition, 4-CMPS modified the NOC-7-, CySNO- or GSNO-induced changes in the gating of the calcium release channel. (i) Activation of the purified calcium release channel by 4-CMPS was observed at subactivating calcium concentrations [24]. NOC-7 (0.3 or 0.6 mM) or GSNO (1–4 mM) did not show any activating effect at a subactivating free calcium concentration of 0.6 μM. However, 4-CMPS activated the calcium release channel in the presence 0.6 mM NOC-7 or 4 mM GSNO (Fig. 3, Table 1C; Fig. 7, Table 8). This finding indicates that NOC-7 or GSNO does not alter the calcium sensitivity of the calcium release channel, whilst oxidation of sulfhydryls of cysteine residues by 4-CMPS increased the apparent calcium affinity of the calcium release channel as described by us previously [24]. Alterations of the apparent calcium affinity of the calcium release channel by the sulfhydryl oxidizing dithiodipyridines (2,2′-DTDP, 4,4′-DTDP) have been shown [26,28]. (ii) The NOC-7-stimulated (Fig. 2) or CySNO-stimulated calcium release channel was maximally activated by 4-CMPS (P_o up to 0.95) in the presence of 20–100 μM activating calcium, which could be due to a 4-CMPS-induced oxidation of activating sulfhydryl(s) of channel protein subunits [24]. Activation was followed by a complete closure of the NOC-7-stimulated channels (Fig. 2). It has been proposed previously that activation and inhibition of the calcium release channel by 4-CMPS [24] or dihydrodipyridines [28] are due to oxidation of different sulfhydryls. (iii) 4-CMPS reversed the CySNO- or GSNO-induced inhibition of the calcium release channel at activating calcium concentrations (Figs. 5 and 7). Inhibitory concentrations of 4 mM GSNO, which reduced P_o by about one order of magnitude, did not prevent the 4-CMPS-induced activation of the calcium release channel (Fig. 7, Table 8). These marked differences between NO donors and the sulfhydryl oxidizing agent 4-CMPS in the

Table 8
Effect of GSNO on single purified skeletal muscle calcium release channels at subactivating Ca^{2+} (0.6 μM) and activation by 4-CMPS

	P_o	n
Control (100 μM Ca)	0.62 ± 0.05	7
Control (0.6 μM Ca)	0.044 ± 0.014	7
GSNO 1 mM	0.058 ± 0.016	6
GSNO 2 mM	0.038 ± 0.015	5
GSNO 4 mM	0.044 ± 0.017	6
GSNO 4 mM/4-CMPS 20–40 μM	0.62 ± 0.12	3

Single channel recordings with the purified calcium release channel were performed in the absence or presence of 1–4 mM GSNO and 4 mM GSNO followed by 20–40 μM 4-CMPS at 0.6 μM Ca^{2+}. Single channel currents were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis*/*trans*). Control and test recordings are from the same channel. Values are means ± S.E.M. for the number of experiments given in parentheses.

modification of the gating properties of the calcium release channel (outlined above) suggest that different sulfhydryls are involved in oxidation of the calcium release channel by 4-CMPS and nitrosylation/oxidation by NO donors assuming that NO donors cause simultaneously nitrosylation/oxidation of the skeletal muscle calcium release channel. Alternatively, the 4-CMPS induced activation of the calcium release channel prior inhibited by CySNO or GSNO could be due to a de-nitrosylation of the calcium release channel by 4-CMPS. To address this question, three experiments have been carried out with 4-CMPS plus GSNO mixed for 1 min and added simultaneously to the *cis* side giving a final concentration of 20–40 μM 4-CMPS and 4 mM GSNO. In all three experiments a transient increase in the open probability was observed when 4-CMPS and GSNO were added simultaneously, followed by an inhibition of the channel activity and finally a channel closure. The open probability increased significantly from 0.50 ± 0.08 to 0.80 ± 0.02 (means ± S.E.M., $n = 3$; $P = 0.02$). These results are not in favor of a de-nitrosylation of *S*-nitrosothiol(s) of the calcium release channel during the 4-CMPS-induced activation of GSNO inhibited channels (Fig. 6). On the other hand, mixtures of 4-CMPS (40 μM–1.2 mM) with 4 mM GSNO kept for 30 s at room temperature before separation of both agents by HPLC (under conditions as described in Section 2 for CySNO isolation) showed that the two compounds interact. The 4-CMPS peak and the GSNO peak of mixtures of both were smaller than observed after separate injection of each compound. Furthermore, a new peak appeared by injecting mixtures of 4-CMPS plus GSNO, whose nature has not been identified, possibly a glutathione-CMPS complex. These results might partly explain the requirement of higher concentrations of 4-CMPS for channel activation in the presence of NO donors and the transient nature of the 4-CMPS-induced activation of the calcium release channel in the presence of high GSNO concentrations. The interaction between 4-CMPS and GSNO observed in the HPLC experiments does not rule out the possibility of a similar interaction between 4-CMPS and *S*-nitrosothiols of the calcium release channel generated by GSNO.

Activation of the calcium release channel by reactive oxygen species, such as H_2O_2, appears to affect sulfhydryls of cysteine residues differently and to oxidize a third type or class of sulfhydryls [37]. H_2O_2 affected sulfhydryls of the calcium release channel from the luminal side and was able to reactivate the 4-CMPS induced reduction in P_o [37]. Furthermore, it was shown that the increase in the open probability by H_2O_2 was associated with intersubunit cross-links, which was reversed by DTT [38]. Interestingly, NOC-9, NOC-15 and SNAP prevented cross-linking of ryanodine receptor monomers by sulfhydryl oxidation with diamide, but high concentrations of NOC-15 induced intersubunit cross-links [18]. SDS-PAGE with the purified skeletal muscle calcium release channel incubated with NOC-7 at conditions similar to those used in the single channel experiments did not show dimer formation or formation of greater protein complexes at concentrations up to 0.6 mM. Intermolecular cross-links appear thus less likely to explain the increase in the open probability by NOC-7 at concentrations used in the present study; the formation of intramolecular cross-links has not been excluded.

In conclusion, the data on single channel current recordings with the purified skeletal muscle calcium release channel show that NO donors activate and inhibit the gating of the calcium release channel in the presence of calcium as sole activator of the calcium release channel. Activation of the calcium release channel was obtained by two classes of NO

donors (*S*-nitrosothiols, NONOate). Comparison of the effects of NO donors with the effects of the sulfhydryl oxidizing compound 4-CMPS suggests that nitrosylation/oxidation by NO donors involve different sulfhydryls of cysteine residues of the calcium release channel as oxidized by the organic mercurial compound.

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